for cardiolipin and phosphatidic acid, respectively, as that for phosphatidylcholine. These differential rates, and also the differential lipid specificities, are difficult to reconcile with the suggestion that the immobilized spin-label lipid component arises from molecules trapped between protein aggregates (Swanson et al., 1980). Indeed, it has previously been shown that trapped spin-labeled lipid has rather different spectral characteristics (Marsh et al., 1978).

Acknowledgments

We thank B. Hirche for her expert technical assistance.

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Structural Requirements for the Binding of Oligosaccharides and Glycopeptides to Immobilized Wheat Germ Agglutinin†

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ABSTRACT: The structural requirements for the interaction of asparagine-linked oligosaccharide moieties of glycoproteins with wheat germ agglutinin (WGA) were investigated by using affinity chromatography on a WGA–Sepharose column. So-called hybrid-type glycopeptides obtained from ovalbumin [Yamashita, K., Tachibana, Y., & Kobata, A. (1978) *J. Biol. Chem. 253*, 3862–3869] were found to have high affinity for WGA–Sepharose, whereas high mannose-type and complex-type glycopeptides were shown to have low affinity. The elution profiles of various glycopeptides modified by glycosidase treatment, Smith periodate degradation, acetolysis, and hydrazinolysis showed that the GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn structure was essential for the binding of glycopeptides to a WGA–Sepharose column. Thus, it was revealed that both the *N*,*N*′-diacetylchitobiose moiety and the β-*N*-acetylglucosaminyl residue linked to C-4 of the β-linked mannose residue contributed to the interaction of the glycopeptide with WGA–Sepharose. The substitution at C-6 of the innermost β-*N*-acetylglucosaminyl residue by an α-fucosyl residue or at C-6 of the β-linked mannose residue by another mannose residue in the above structure reduced the affinity of glycopeptides for the column.

Wheat germ agglutinin (WGA)[1] has become an important tool for the isolation and characterization of a variety of glycoconjugates, especially glycoproteins. The binding specificity of WGA has been investigated in several laboratories. *N*-Acetylglucosamine and its β(1–4) oligomers have been found to be potent inhibitors of WGA in agglutination (Burger & Goldberg, 1967; Allen et al., 1973; Krug et al., 1973; Lotan et al., 1975), in mitogenic stimulation of lymphocytes (Brown

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[1] Abbreviations used: WGA, wheat germ agglutinin; OA, ovalbumin; UA, porcine thyroglobulin unit A glycopeptide; UB, porcine thyroglobulin unit B glycopeptide.

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et al., 1976), and in precipitation of glycoconjugates (Lotan et al., 1975; Goldstein et al., 1975). The interaction of WGA with another saccharide, *N*-acetylneuraminic acid, was also reported (Burger & Goldberg, 1967; Cuatrecasas, 1973; Bhavanandan & Katlic, 1979; Peters et al., 1979), and several sialoglycoproteins and sialoglycopeptides were isolated by using affinity chromatography (Adair & Kornfeld, 1974; Bhavanandan et al., 1977) on a column of WGA.

Recently, the structural requirements of an oligosaccharide or a glycopeptide for the binding to concanavalin A–Sepharose were elucidated (Ogata et al., 1975; Krusius et al., 1976), and the affinity chromatography on a concanavalin A–Sepharose column has become a useful technique for structural studies of glycopeptides and oligosaccharides and their fractionation (Hodges et al., 1979; Yamamoto et al., 1981). WGA–Sepharose has also been widely used for the purification of many glycoproteins (Lotan & Nicolson, 1979). The purpose of the present investigation was to study the structural basis of glycopeptides for the binding to the WGA–Sepharose column. It has been presumed that the *N,N'*-diacetylchitobiose moiety commonly present in the core region of asparagine-linked oligosaccharides of glycoproteins is recognized by WGA–Sepharose. We found that the so-called hybrid-type glycopeptides prepared from ovalbumin (Yamashita et al., 1978) had higher affinity for this column than high mannose-type or complex-type glycopeptides, although all glycopeptides used in the present study had an *N,N'*-diacetylchitobiose moiety in their core region. Furthermore, we elucidated the essential sugar structure for the binding of the glycopeptides to the WGA–Sepharose column.

Materials and Methods

Preparation of WGA. Wheat germ was purchased from Sigma Chemical Co. (St. Louis, MO). Crude WGA was prepared by delipidation with acetone, extraction with 0.05 M HCl, and fractionation with ammonium sulfate as described by Nagata et al. (1974). After dialysis against 0.05 M sodium phosphate buffer, pH 7.0, containing 0.25 M sodium chloride, the solution was heated at 58 °C for 15 min to inactivate β-*N*-acetylhexosaminidase which might hydrolyze the affinity adsorbent (Marchesi, 1972). After centrifugation, the supernatant was subjected to affinity chromatography on a column of chitotriose–Sepharose 4B prepared by the method of Matsumoto et al. (1980). The column was washed with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.25 M sodium chloride until the absorbance at 280 nm became approximately 0.01, and WGA was then eluted with 0.1 M acetic acid.

Conjugation of WGA to Sepharose 4B. Purified WGA was coupled to Sepharose 4B according to the method of Matsumoto et al. (1980). The amount of WGA bound to Sepharose was estimated to be approximately 3.5 mg/mL of gel by subtraction of the amount of unbound protein in the supernatant and the wash after the coupling reaction. Protein was determined by the method of Lowry et al. (1951).

Affinity Chromatography on a WGA–Sepharose Column. The radioactively labeled sample [(2–5) × 10^3 cpm, 0.1–0.25 nmol] in a volume of 50 μL was applied to a WGA–Sepharose column (1.5 mL) equilibrated with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M sodium chloride and allowed to stand at room temperature for 1 h. The column was then eluted with the same buffer, followed by 0.1 M *N*-acetylglucosamine in the same buffer. Elution was performed at a flow rate of 3 mL/h, and fractions of 1.5 mL were collected. Recoveries of radioactivity in all experiments were more than 95%.

Glycopeptides and Oligosaccharides. Chitin oligosaccharides were prepared by limited hydrolysis of chitin according to the method of Rupley (1964). Ovalbumin glycopeptides GP-I, GP-II-A, and GP-II-B were prepared as described by Tai et al. (1975) and Yamashita et al. (1978). Porcine thyroglobulin glycopeptides were prepared as described previously (Tsuji et al., 1981; Yamamoto et al., 1981). Unit A type (high mannose-type) and unit B type (complex-type) glycopeptides were separated by DEAE-Sephadex column chromatography. Unit B type glycopeptide was used after α-fucosidase treatment. Tryptic fragment T1 of glycophorin A was prepared from human erythrocytes by the method of Tomita et al. (1978). The asparagine-linked glycopeptide fraction was obtained by alkaline borohydride treatment (Fukuda & Osawa, 1973) of the tryptic fragment T1 of glycophorin A, followed by exhaustive Pronase digestion. After removal of sialyl residues by mild acid hydrolysis with 0.05 M H_2SO_4 at 80 °C for 1 h, the major glycopeptide (GP I-N-3) was isolated by chromatography on columns of Sephadex G-25 and *Ricinus communis* agglutinin–Sepharose. Compositional analysis showed that the sugar chain of this glycopeptide corresponds to the oligosaccharide analyzed in the previous studies (Yoshima et al., 1980; Irimura et al., 1981). The structures of these standard glycopeptides were confirmed to be those reported in the original papers by the compositional analysis and methylation study and are given in Figure 1.

Modification of Glycopeptides. Smith periodate degradation was carried out as described previously (Yamamoto et al., 1981). Acetolysis was performed by the method of Kocourek & Ballou (1969) as described previously (Tsuji et al., 1981). Hydrazinolysis was carried out according to the method of Fukuda et al. (1976). α-Mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase were purified from jack bean meal (Sigma Chemical Co.) by the method of Li & Li (1972). Neuraminidase from *Arthrobacter ureafaciens* was purchased from Nakarai Chemical Co. (Kyoto, Japan). α-L-Fucosidase from *Charonia lampas* was purchased from Seikagaku Kogyo Ltd. (Tokyo, Japan).

Glycopeptides were digested at 37 °C with glycosidase (0.1–0.5 unit) in 0.1 mL of the appropriate buffers under a toluene layer for 24–48 h followed by heating at 100 °C for 3 min to stop the reaction. Then each reaction mixture was passed through small columns of Dowex 50W-X8 (H^+ form; 0.8 mL) and Bio-Rad AG-3 (OH^- form; 0.8 mL). Recoveries of the modified glycopeptides were usually more than 90% of the theoretical values. Digestions with β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were carried out in 50 mM sodium acetate buffer (pH 4.0). For digestion with α-L-fucosidase, 0.1 M sodium citrate–0.1 M sodium phosphate buffer (pH 4.0) containing 0.5 M NaCl was used.

Labeling of Glycopeptides and Oligosaccharides. The radioactive label on the glycopeptide was introduced by acetylation with [^{14}C]acetic anhydride (30.0 mCi/mmol, The Radiochemical Centre, Amersham, England) by the method of Tai et al. (1975). The labeled glycopeptide was purified by gel filtration on a column of Sephadex G-25. An oligosaccharide was labeled on the reducing terminal residue by reduction with NaB^3H_4 (250 mCi/mmol, New England Nuclear, Boston, MA) by the method of Takasaki & Kobata (1978).

High-Voltage Paper Electrophoresis. High-voltage paper electrophoretic analyses of labeled glycopeptides and oligosaccharides were carried out with a Fujiox high-voltage electrophoresis apparatus (Fujiriken, Tokyo, Japan) on Whatman No. 1 paper in pyridine–acetic acid–water (3:1:387)

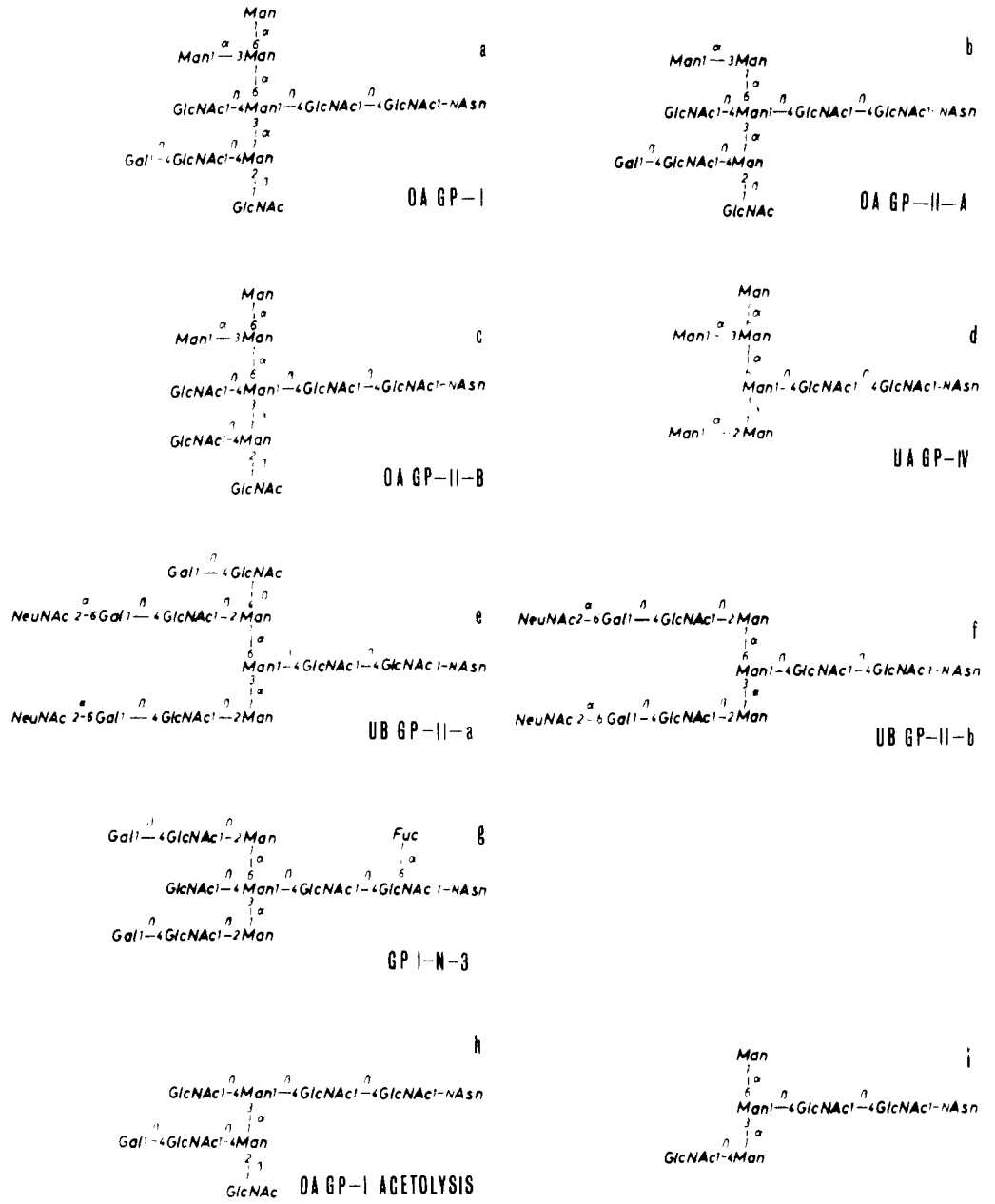

FIGURE 1: Structures for a series of *N*-acetylated glycopeptides obtained from ovalbumin, porcine thyroglobulin, and human glycophorin A and for acetolysis or Smith degradation products of ovalbumin glycopeptide GP-I. (a–c) Ovalbumin (OA) glycopeptides GP-I, GP-II-A, and GP-II-B. (d) Porcine thyroglobulin unit A (UA) glycopeptide GP-IV. (e and f) Porcine thyroglobulin unit B (UB) disialyl glycopeptides GP-II-a and GP-II-b. (g) Human glycophorin A asparagine-linked glycopeptide I-N-3. (h) Acetolysis product of ovalbumin glycopeptide GP-I. (i) Smith degradation product of ovalbumin glycopeptide GP-I.

at the potential of 70 V/cm for 1.5 h according to the method of Tai et al. (1975).

Composition and Methylation Analyses. The carbohydrate compositions of glycopeptides and oligosaccharides were analyzed by using a gas–liquid chromatograph with a column (0.3 × 100 cm) of 0.05% ECNSS-M; the carbohydrates were chromatographed as alditol acetates after hydrolysis with 2 M HCl at 100 °C for 3 h or with 4 M HCl at 100 °C for 6 h. Amino acids in the glycopeptides were determined with an amino acid analyzer (Hitachi Model 835; Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl at 110 °C for 24 h.

Glycopeptides were methylated by the method of Hakomori (1964). After hydrolysis, reduction, and acetylation, the alditol acetates of partially methylated sugars were analyzed with a gas chromatograph–mass spectrometer (Shimadzu-LKB Model 9000) according to the method of Stellner et al. (1973) as described previously (Yamamoto et al., 1981).

Results

Figure 2 shows the behavior of chitin oligosaccharides reduced with NaB^3H_4 on a column of WGA–Sepharose. Although *N,N'*-diacetylchitobiose alditol was not bound to the column (Figure 2a), *N,N',N''*-triacetylchitotriose alditol and *N,N',N'',N'''*-tetraacetylchitotetraose alditol were retained on the column and eluted with the buffer containing 0.1 M and 0.2 M *N*-acetylglucosamine, respectively. This showed that the affinity of chitin oligosaccharide alditol for the WGA–Sepharose column increased with the increasing number of *N*-acetylglucosamine residues. This was in good agreement with the data of the precipitation inhibition experiments reported by Goldstein et al. (1975).

Then, we examined several asparagine-linked glycopeptides, which were classified into high mannose-type, complex-type, or hybrid-type glycopeptides, for the binding to a WGA–Se-

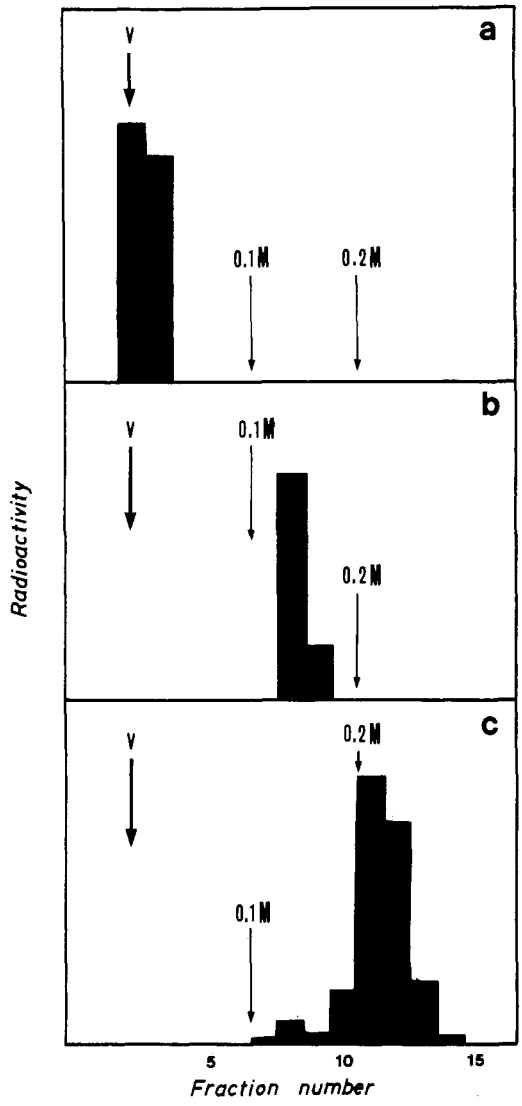

FIGURE 2: Affinity chromatography of NaB^3H_4-reduced *N*-acetylglucosamine oligomers on a WGA–Sepharose 4B column. Experimental details are given under Materials and Methods. Elution was performed with 10 mM phosphate buffer, pH 7.3, containing 0.15 M NaCl, followed by the same buffer containing 0.1 and 0.2 M *N*-acetylglucosamine at a flow rate of 3 mL/h, and 1.5-mL fractions were collected. An arrow, V, indicates the void volume fraction. The other arrows indicate the positions where the buffer was changed. (a) Elution profile of GlcNAcβ1–4GlcNAcol. (b) Elution profile of GlcNAcβ1–4GlcNAcβ1–4GlcNAcol. (c) Elution profile of GlcNacβ1–4GlcNAcβ1–4GlcNAcβ1–4GlcNAcol.

pharose column. When a high mannose-type glycopeptide, $(Man)_6$-GlcNAc-GlcNAc-Asn (Figure 1d) obtained from porcine thyroglobulin, was applied to a column of WGA–Sepharose, it was recovered without retardation (Figure 3a). The complex-type glycopeptides, $(NeuAc)_2$-$(Gal)_2$-$(GlcNAc)_2$-$(Man)_3$-$(GlcNAc)_2$-Asn (Figure 1f) and $(NeuAc)_2$-$(Gal)_3$-$(GlcNAc)_3$-$(Man)_3$-$(GlcNAc)_2$-Asn (Figure 1e), were also eluted in the void volume fraction from the column (Figure 3b). In these experiments, therefore, no interaction was observed between *N*-acetylneuraminic acid residues in these complex-type glycopeptides and the WGA column. The same results were obtained in the cases of the complex-type glycopeptides treated with neuraminidase or neuraminidase plus β-galactosidase (data not shown). On the other hand, the hybrid-type glycopeptides, ovalbumin glycopeptides GP-I, GP-II-A, and GP-II-B (Figure 1a–c), were found to be retarded on a WGA–Sepharose column (Figure 3c,d). This indicates that the hybrid-type glycopeptides have higher affinity for the column than the other two types of glycopeptides. In order to clarify the structural elements responsible for the high affinity of hybrid-type glycopeptides for the column, the glycopeptides modified by acetolysis or Smith periodate degradation were applied to the column. The structures of the products after acetolysis (Figure 1h) or Smith periodate degradation (Figure 1i) of ovalbumin glycopeptide GP-I had already been reported by Yamashita et al. (1978) and were confirmed in this study by the compositional analysis and methylation study. After removal of three α-mannosyl residues on the branch arising from C-6 of the β-mannosyl residue by acetolysis, the glycopeptide obtained was retained on a WGA–Sepharose column, indicating the increase of its affinity for the column (Figure 4a). After removal of these α-mannosyl residues by digestion of glycopeptide GP-I with α-mannosidase, the glycopeptide obtained gave the same result (data not shown). On the other hand, Smith periodate degradation of the glycopeptide GP-I was found to diminish the binding ability to the column (Figure 4b). This may be due to the removal of a β-*N*-acetylglucosamine residue attached to C-4 of the β-linked mannose residue, which is one of the special structural features on hybrid-type glycopeptides. Since the importance of the β-*N*-acetylglucosaminyl residue linked to the β-linked mannose residue for the interaction between glycopeptides and WGA–Sepharose was suggested from the foregoing results, two glycopeptides, GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn and Manβ1–4GlcNAcβ1–4GlcNAc-Asn, were then examined. GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn was prepared from *N*-^{14}C-acetylated ovalbumin glycopeptide GP-II-B (Figure 1c) by successive treatments with β-*N*-acetylhexosaminidase (50 milliunit) and α-mannosidase (0.5 unit). The glycopeptide containing a tetrasaccharide was purified by paper electrophoresis, and its structure was ascertained by the compositional analysis and by paper electrophoresis in which it comigrated after β-*N*-acetylhexosaminidase digestion with Manβ1→4GlcNAcβ1→4GlcNAc-Asn prepared by exhaustive α-mannosidase digestion of porcine thyroglobulin unit A type glycopeptide (Figure 1d; Tsuji et al., 1981). The β-*N*-acetyl-

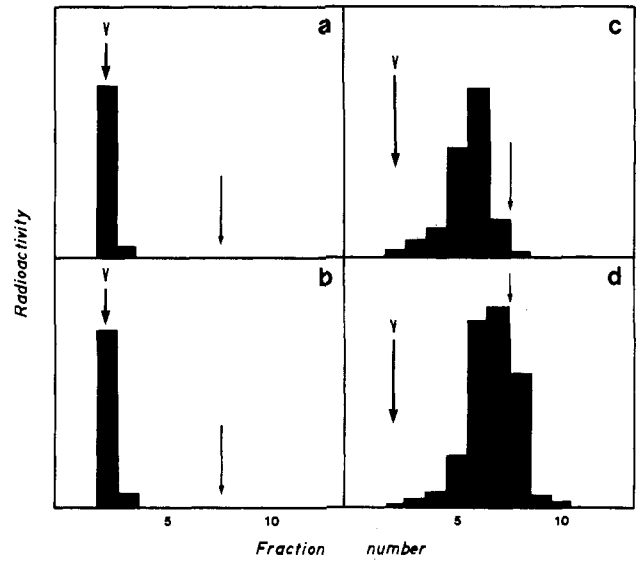

FIGURE 3: Affinity chromatography of asparagine-linked glycopeptides on a WGA–Sepharose 4B column. Experimental details are given in the legend to Figure 2. An arrow, V, indicates the void volume fraction, and the other arrow indicates the position where elution with the buffer containing 0.1 M *N*-acetylglucosamine was started. (a) Elution profile of UA GP-IV. (b) Elution profile of UB GP-II-a and GP-II-b. (c) Elution profile of OA GP-I. (d) Elution profile of OA GP-II-A.

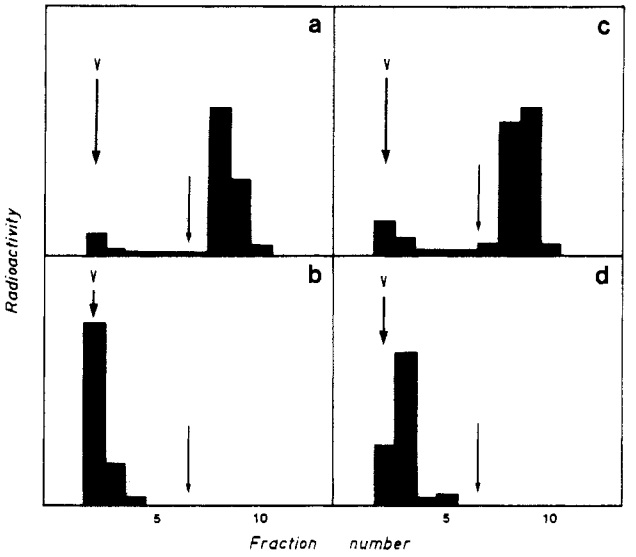

FIGURE 4: Affinity chromatography of ovalbumin glycopeptide GP-I after acetolysis or Smith degradation and of GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn and Manβ1–4GlcNAcβ1–4GlcNAc-Asn. Experimental details and symbols are the same as in the legend to Figure 3. (a) Elution profile of acetolysis product of OA GP-I. (b) Elution profile of Smith degradation product of OA GP-I. (c) Elution profile of GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn. (d) Elution profile of Manβ1–4GlcNAcβ1–4GlcNAc-Asn.

glucosaminyl residue linked to C-4 of the β-linked mannose residue was thus found to be resistant to this β-*N*-acetylhexosaminidase treatment. As expected, the glycopeptide, GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn, prepared was retained on a WGA–Sepharose column (Figure 4c). Manβ1–4GlcNAcβ1–4GlcNAc-Asn (Figure 1d) was, however, found to have no ability to bind to the column. These results indicate that the *N*-acetylglucosamine residue located at C-4 of the β-linked mannose residue greatly contributes to the interaction of a glycopeptide with a WGA–Sepharose column.

We then examined the effect of the modification in the *N*,*N*′-diacetylchitobiose moiety at the reducing end of sugar chains upon the binding to WGA–Sepharose. The oligosaccharide moiety liberated from ovalbumin glycopeptide GP-I (Figure 1a) by hydrazinolysis according to the method of Fukuda et al. (1976) was radioactively labeled by reduction with NaB^3H_4 and converted to an oligosaccharide having *N*-acetylglucosaminitol at the reducing end. The oligosaccharide alditol was found to pass through the WGA–Sepharose column without retardation (data not shown). Although the unlabeled reducing oligosaccharide was not tested for the binding to the WGA–Sepharose column owing to the limited availability of GP-I, the above result suggests that an intact *N*-acetylglucosamine residue at the reducing end is necessary for the binding to a WGA–Sepharose column, because reducing *N*,*N*′,*N*″-triacetylchitotriose can bind to the WGA–Sepharose column, indicating that the asparagine residue in GP-I does not contribute to the binding property of the glycopeptide.

We prepared another glycopeptide (Figure 1g) with a β-*N*-acetylglucosaminyl residue linked to C-4 of the β-linked mannose residue from human erythrocyte glycophorin A. The structure of the sugar chain of this glycopeptide was reported in a previous paper (Irimura et al., 1981). No interaction of the intact glycopeptide with a WGA–Sepharose column was observed (Figure 5a). After removal of an α-fucosyl residue attached to C-6 of the innermost *N*-acetylglucosamine residue by α-fucosidase treatment this glycopeptide, however, could

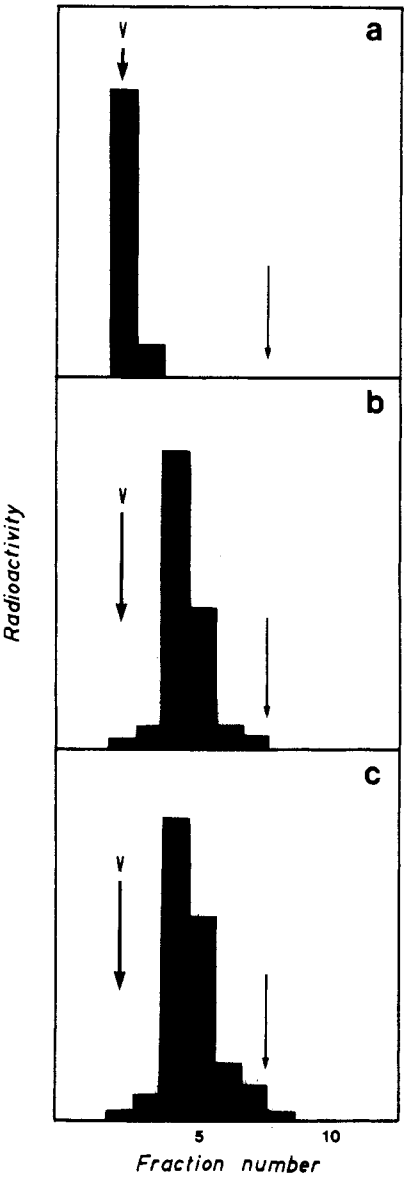

FIGURE 5: Affinity chromatography of human glycophorin A asparagine-linked glycopeptide GP I-N-3 and of its exoglycosidase digests on a WGA–Sepharose 4B column. Experimental details and symbols are the same as in the legend to Figure 3. (a) Elution profile of GP I-N-3, prepared from human glycophorin A. (b) Elution profile of GP I-N-3 after α-fucosidase treatment. (c) Elution profile of GP I-N-3 after treatment with a mixture of α-fucosidase and β-galactosidase.

be retarded by the column (Figure 5b). This shows that an α-fucosyl residue attached to an *N*-acetylglucosamine residue in the core portion inhibits the interaction of the glycopeptide with WGA–Sepharose probably due to steric hindrance. Further treatment of this glycopeptide with β-galactosidase did not result in an increase of affinity for the column (Figure 5c).

From the foregoing series of experiments, we concluded that the following structural features were essential for asparagine-linked glycopeptides to bind to a WGA–Sepharose column: (1) a complete *N*,*N*′-diacetylchitobiose structure present in the core portion; (2) an *N*-acetylglucosamine residue attached to an inner β-linked mannose.

Discussion

It is known that the binding of sialic acid free asparagine-linked oligosaccharide moieties of glycoproteins to WGA can

be attributed to the internal *N*-acetylglucosamine residues present in the core portion. In the present study, we found by using an affinity chromatographic technique that the hybrid-type glycopeptides prepared from ovalbumin had higher affinity for WGA–Sepharose than high mannose-type or complex-type glycopeptides although all the glycopeptides used contained an *N,N'*-diacetylchitobiose moiety. This observation is in good agreement with the one that ovalbumin is a powerful inhibitor of hemagglutination caused by WGA. It is concluded that the strong interaction between hybrid-type glycopeptides and WGA–Sepharose is due to the presence of a nonreducing terminal *N*-acetylglucosamine residue attached to C-4 of the β-linked mannose residue. This conclusion was clearly verified by the fact that GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn could bind to a WGA–Sepharose column but lost the ability to bind to the column after removal of a terminal *N*-acetylglucosamine residue. Allen et al. (1973) and Monsigny et al. (1978) reported that the binding site of WGA consisted of three or four subsites with different specificities. Each of the subsites A, B, and C can bind an *N*-acetylglucosamine residue, and subsite D can accommodate an aglycon of the saccharide. The tetrasaccharide, GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc, seemed to fit in subsites A, B, C, and D. It seems that the substitution at C-6 of the reducing terminal *N*-acetylglucosamine residue by an α-fucosyl residue or the reduction of the reducing terminus makes it difficult for the other *N*-acetylglucosamine residues to interact with subsites A, B, and C. According to the assumption made by Allen et al. (1973), in subsite C the presence of a derivative of *N*-acetylglucosamine with a free 3-hydroxy group is essential, but for binding to subsite B, a free 3-hydroxy group is not essential. Their suggestion is consistent with our present results. Actually, in this study, the binding ability was increased by the removal of three α-mannosyl residues on the branch arising from C-6 of the β-linked mannose of ovalbumin glycopeptides GP-I by acetolysis or α-mannosidase treatment, but the substitution at C-3 of the β-mannosyl residue had no or little effect on the binding.

In the present investigation, the interaction of the glycopeptides containing *N*-acetylneuraminic acid residues with WGA–Sepharose was not observed. The presence of clustering sialyl residues may be necessary for sialo glycoconjugates to bind to a WGA–Sepharose column as suggested by Bhavanandan et al. (1977). Human erythrocyte glycophorin A was reported as a receptor for WGA (Adair & Kornfeld, 1974). Since the interaction of the desialylated asparagine-linked glycopeptide with WGA–Sepharose was not as strong as expected, the interaction of glycophorin A with WGA may be due to clustering sialo oligosaccharide groups on the peptide backbone.

Recently, a number of studies suggested that cell membrane glycoproteins play an important role in many biological phenomena. In most cases, however, it is difficult to investigate the structure of these glycoproteins because of the limited amounts of samples available. The finding in the present work will be useful for the purification and fractionation of glycoconjugates. Furthermore, some information on their structures could also be obtained by monitoring the elution profiles from an affinity column of WGA–Sepharose, when the structural analyses cannot be performed because of the lack of samples.

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